OBSERVATIONS ON *ENTAMOEBA HISTOLYTICA*

I. DEVELOPMENT OF CYSTS, EXCYSTATION, AND DEVELOPMENT OF EXCYSTED AMOEBAE, *IN VITRO*

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PLATES XXI-XXIV

For many years it has been generally accepted that the cysts of *Entamoeba histolytica* will hatch only after they have been subjected to the action of the gastric and pancreatic juices, or to that of the pancreatic juice alone. Study of the literature suggests that this hypothesis is probably mainly based on the observations of Ujihara (1914), of Penfold, Woodcock, and Drew (1916), of Chatton (1917), and of Cutler (1919). Ujihara records that cysts after incubation with gastric juice for 24 hours at 37° C. remained for the most part undigested, but that pancreatic juice was much more active. Penfold, Woodcock, and Drew write as follows : ' As excysting agents we have tried pepsin in an acid medium, bile, and pancreatic extract, either alone, consecutively, or together, as appeared indicated, but the only success we have had has been with pancreatic extract used alone.' Cutler states that ' if a solution of " liquor pepticus " is first allowed to act on the cysts for a short time, followed by a similar solution of " liquor pancreaticus," a very large proportion of the treated cysts react.' Chatton's experiments were of a different type, but permit of similar conclusions ; he fed cats with *E. histolytica* cysts, and sacrificed the animals after periods varying from $3\frac{1}{2}$ to 17 hours ; careful examination of the contents of different portions of the alimentary canal caused him to draw the conclusions that cysts pass through the stomach without alteration, except for the digestion of chromidial bars, and that excystation takes place in the small intestine.

It is remarkable that these workers have either overlooked or ignored the very interesting paper of Darling (1913). Chatton, it

is true, refers to his work, but offers no comment upon it. Darling describes the gradual disappearance of cysts from moist chamber preparations of heavily-infected faeces, and also the development of amoebulae within the cysts, and their emergence. This statement is so definite and based upon apparently such careful observations that it is curious it should have escaped comment from those who later worked on the same subject. Probably there are several reasons for this, most influential of which was the general pre-conception that the cysts must be swallowed before they can develop ; another consideration was, doubtless, the possibility that Darling's preparations were—as so often happens in the tropics—contaminated with free-living amoebae.

However this be, the fact remains that Darling's work seems to have been more or less ignored and the view generally accepted that *Entamoeba histolytica* only excysts when it has been swallowed and subjected to the influence of the digestive juices. Dobell and O'Connor (1921), in their book on *The Intestinal Protozoa of Man,* after considering previous work on the subject conclude that ' it is certain that the cysts never hatch in the colon, where they are found, or outside the body.'

In 1924, however, an important piece of work was published by Sellards and Theiler, which indicated that *Entamoeba histolytica* cysts would hatch when injected intra-rectally in kittens, and their work, which was very carefully controlled, has quite recently been confirmed by Hoare (1925).

This observation is naturally of considerable importance and re-opens the whole subject regarding the conditions necessary for the excystation of *Entamoeba histolytica,* and possibly has some bearing on the factors concerned in relapse production in amoebic dysentery.

With the object of throwing further light on the matter we have conducted, during the past six months, a large number of experiments, the nature of which is described below.

The work commenced with the inoculation of a couple of tubes of Locke-egg-serum medium (Boeck, 1924, and Boeck and Drbohlav, 1925) with a small amount of a human faeces containing numerous cysts of *Entamoeba histolytica,* and incubating at 37° C. On examining the tubes next day, we were surprised to find large numbers of vegetative amoebae morphologically indistinguishable

from *Entamoeba histolytica*. From these tubes others were sub-inoculated, and it was found that the strain was just as readily maintained for many generations, as were those originating from the vegetative forms in acute dysenteric stools.

As no vegetative amoebae were discovered in the stools at the time of inoculation, it appeared that those present in such large numbers in the culture the following day, were derived from the cysts. Numerous similar experiments with the freshly-passed faeces of the same patient, and also with those stored at laboratory temperature for three or four days, gave comparable results, as did, likewise, experiments performed with the stools of six or seven other patients which contained the cysts of *Entamoeba histolytica*.

We can therefore accept as a fact that *Entamoeba histolytica* readily excysts on L.E.S. (Locke-egg-serum) medium at 37° C.; and, as in 24 hours a plentiful growth of vegetative forms can be obtained, the phenomenon can be used with great advantage as a ready means of obtaining a supply of active amoebae.* For this purpose we found the following procedure to be well-adapted:—

(i) A mass of faeces about the size of a walnut is ground up in a small mortar with a little water, and the emulsion thus formed thoroughly shaken with 500 c.c. of water, and then poured into a tall glass cylinder and allowed to stand for about fifteen minutes. This period is sufficient to permit all the larger and heavier faecal particles to fall to the bottom of the cylinder, and a small scum consisting of very light matter to accumulate at the surface: the scum is removed, and the bulk of the fluid then withdrawn by means of a syphon, leaving only the bottom inch or so and the precipitated mass, which are thrown away. The fluid which has been decanted is now placed in another tall cylinder and allowed to stand over-night, by which time all the cysts, with a certain amount of faecal material, will have settled to the bottom of the cylinder.

The supernatant fluid is again withdrawn, by means of a syphon, and rejected, and the precipitate containing the cysts washed several times by shaking up with water and centrifuging. By this means a deposit is finally obtained which consists of only the finest faecal particles, with relatively few bacteria, and the majority of the cysts. If it be desired to hasten the process, the original fluid, which has been decanted off the coarse faecal deposit after standing fifteen minutes, can be centrifuged immediately, instead of being allowed to stand all night, and the deposit washed repeatedly with water as described above.

If the cysts in the original faeces are scanty, or if for any purpose a particularly high concentration of cysts is required, with relatively

*Whilst this paper was in preparation, an article by St. John (1926) has appeared, in which it is briefly stated that motile *Entamoeba histolytica* were obtained by sowing on L.E.S. medium a 48-hour old specimen of faeces containing a few *Entamoeba histolytica* cysts; a similar result was also got from the same specimen after it had been kept eight days in the ice-chest.

very little faecal material, the following modification of the above procedure has been found to serve most admirably :—

(ii) As before described, the faeces is ground up in a small mortar with water, and the emulsion shaken up with 500 or 1000 c.c. of water, poured into a tall glass cylinder, and allowed to stand for fifteen minutes to get rid of the coarser faecal material. The supernatant fluid is withdrawn and either centrifuged or allowed to stand over-night in a cylinder : the deposit is then shaken up thoroughly with a solution of cane sugar in water, of a sp. gr. of about 1080, and centrifuged at high speed. This procedure results in separation of the vast majority of the cysts from the remaining faecal material, the faeces being precipitated, and the cysts floating in the supernatant fluid, which is withdrawn, diluted with about four times its volume of water, and again centrifuged at high speed ; by this means a small deposit is obtained consisting of great numbers of cysts in a relatively minute quantity of faecal material. The deposit is then washed several times with water, to get rid of all traces of sugar and the majority of the remaining bacteria.

Washed concentrated suspensions of cysts prepared by this method were found to be remarkably satisfactory for obtaining cultures of *Entamoeba histolytica ;* the relatively few bacteria which such suspensions contained, as compared with the original faeces, enabled the excysting amoebae to become well-established before the growth of bacteria swamped them.

Careful comparison of the results obtained from cultures of suspensions of cysts, prepared by each of the above methods, has failed to reveal any indication that the concentrated sugar solution has a deleterious effect on the cysts.

The culture tubes should not be placed vertically in the incubator, but should lie so that the top of the egg slope is practically horizontal. In our experience the best way of examining them is to shake the tube vigorously, so that the fluid and solid material (bacteria, amoebae, and débris) on top of the egg slope are thoroughly mixed, and then with a pipette to remove about 1 c.c. and centrifuge it in a warm tube for about half-a-minute ; most of the supernatant fluid is then withdrawn and the deposit stirred up with the remainder and examined, preferably in a hot microscope chamber, when the amoebae can readily be detected in large numbers. This procedure appears to us to be much more reliable and satisfactory than merely sampling the bottom of the unshaken culture tube with either a pipette or platinum loop. For sub-inoculation we, likewise, shake the culture tube, remove a few drops with a pipette, and inoculate on to a warm fresh medium ; the best results are obtained when subculture is performed daily.

CHANGES OCCURRING IN CYSTS IN CULTURE TUBES

Before discussing the changes in the cysts which take place on incubation at 37° C. on L.E.S. medium, we should note that the cysts passed by different patients, and even by the same patient at different times, vary greatly in appearance, especially in respect of the number of nuclei they contain, and of their chromatoid bodies and glycogen content. For example, on one occasion the cysts may be practically all uninucleate with much glycogen, and but few of them containing chromatoid bodies, whereas on another occasion the vast majority may be quadrinucleate with chromatoid bodies and but little glycogen. We shall, however, return to this subject in a later communication and here confine ourselves to the changes occurring in the culture tubes.

Multiplication of nuclei. Undoubtedly the most striking change is the rapid multiplication of the nuclei in the uni- and bi-nucleate cysts, so that within a few hours practically all the cysts have become quadrinucleate. This is well illustrated in an experiment, details of which are given in Table I. A washed concentrated suspension of *E. histolytica* cysts was quickly made from the freshly-passed faeces of a patient, and inoculated on L.E.S. medium. The cysts in the original suspension, and in samples of the culture taken at short intervals, were carefully examined in iodine and the percentage of uni-, bi-, and quadri-nucleates ascertained. It should be here noted that although a few trinucleate cysts were regularly found, we have not thought it necessary to classify them separately, and in this work they have always been grouped with the quadri-nucleates.

TABLE I.

Showing the conversion of uni- and bi-nucleate cysts into quadri-nucleate cysts during incubation at 37° C. on L.E.S. medium.

Type of cyst	Original suspension	After incubation at 37° C. on L.E.S. medium			
		2 hours	2½ hours	4½ hours	8 hours
Uninucleate	42	24	8	8	1
Binucleate	18	13	9	4	0
Quadrinucleate	27	55	75	53	65
Nuclei indistinct, or cysts shrunken or granular	13	8	8	3	11
Vegetative amoebae	...	...	...	32	23

It will be seen from this table that incubation resulted in a steady decline in the percentage of uninucleate, and a corresponding increase in that of quadrinucleate, cysts: within about $4\frac{1}{2}$ hours, definite numbers of vegetative amoebae began to appear in the cultures. This rapid development is particularly interesting in view of the generally preconceived notion that the cysts do not develop outside the body; e.g., Dobell (1919), writing of *Entamoeba histolytica* cysts states 'Those containing less than four nuclei never develop to maturity outside the body, and usually die much sooner than the mature cysts. Even cysts with dividing nuclei do not complete their nuclear divisions. Spindle-figures and other stages arrested in division can be seen to remain unchanged within the cysts until degeneration takes place.'

In passing, it might be noted that in an appreciable proportion of cysts, both before and after culture, the nuclei were indefinite and sometimes invisible and the cytoplasm appeared granular; in short, the cysts conveyed the impression that they were dead or degenerating.

We have been unable to discover any evidence that, during the development of the cysts, autogamy, such as has been described by Wenyon (1907), in the case of *Entamoeba muris*, occurs. We have observed in the living cysts the multiplication of the nucleus with the formation of four daughter nuclei, the agglomeration of the nuclei, and the excystment of the 4-nucleated amoeba. We have seen nothing suggestive of fusion of the daughter nuclei either in living or stained preparations. In a recent letter, Dr. Wenyon informs us that he now believes he was mistaken regarding the occurrence of autogamy in the cysts of *Entamoeba muris*, and that he is stating so in his forthcoming book on Protozoology.

On various occasions, what appeared to be particles of chromatin were seen to be extruded from the nucleus during development of the cysts. Such appearances are illustrated in Pl. XXI, figs. 3 and 4, and it seems probable that a reduction of chromatin is associated with nuclear division.

So far as we have been able to ascertain, the following changes occur in nuclear division. The karyosome appears to lose its definite outline and to become fragmented; this is possibly associated with extrusion of chromatin particles from the nucleus (Pl. XXI, fig. 3);

two daughter karyosomes then make their appearance, and the nucleus elongates in a spindle-shaped manner and appears to be traversed in a longitudinal direction by numerous fibrils arranged irregularly (Pl. XXI, figs. 4 to 7); a constriction then appears about the equator and the ends become rounded, the nucleus assuming a dumb-bell-shaped appearance; finally, the neck constricts and division is completed (Pl. XXI, figs. 8 to 10).

Glycogen content. Another very definite change seen in the cysts during culture was the rapid disappearance of glycogen. When first passed practically all the uninucleate cysts contained a more or less large mass of glycogen which stained deeply with iodine, and to some extent frequently masked the nucleus: this was seen also in the binucleate cysts, but to a much less extent; the quadrinucleate cysts rarely contained any appreciable quantity of glycogen, and as a result stained much less deeply than the uninucleate cysts, and the nuclei were clearly visible.

Chromatoid bodies. Careful examination of freshly-passed faeces showed that these bodies were comparatively rarely present in the uninucleate cysts, but were more commonly found in the bi- and quadri-nucleates. Study of cultures indicates that there is a definite cycle in their development; they were found in a much larger proportion of cysts after a few hours' incubation than before, but finally, in still older cultures, in which vegetative amoebae were beginning to appear, they were greatly diminished in number, and there is reason to believe that in the vast majority of cysts the chromatoid bodies disappear before excystation.

The changes during culture undergone by the cysts in respect of the number of nuclei, glycogen content, and chromatoid bodies, are well shown in an experiment, details of which are set forth in Table II.

This table shows clearly the development of the nuclei during culture, the quadrinucleates which in the original faeces comprised 40 per cent. of the total cysts, after $5\frac{1}{2}$ hours' incubation on L.E.S. medium at 37° C. had (including the quadrinucleate amoebae) increased to 88 per cent.; the progressive disappearance of glycogen, and the initial increase, and subsequent decrease, in the percentage of cysts containing chromatoid bodies are equally apparent.

TABLE II.

Showing the changes undergone by *Entamoeba histolytica* cysts during cultivation on L.E.S. medium at 37° C., in respect of the number of nuclei, glycogen content, and chromatoid bodies.

No. of hours incubated		Number of nuclei			Nuclei indistinct, or cysts shrunken or granular	Vegetative amoebae
		1	2	4		
Original faeces ...	Glycogen only	21	9	7	...	...
	Chromatoids only ...	1	2	6	...	...
	Glycogen + chromatoids	4	6	8	...	...
	Neither	8	8	19	...	...
	Total	34	25	40	1	0
1½ hours	Glycogen only	7	3	3	...	...
	Chromatoids only ...	6	6	33	...	...
	Glycogen + chromatoids	3	2	4	...	...
	Neither	9	4	16	...	...
	Total	25	15	56	4	0
3¾ hours	Glycogen only	0	0	0	...	...
	Chromatoids only ...	5	12	39	...	...
	Glycogen + chromatoids	0	0	0	...	...
	Neither	5	3	23	...	...
	Total	10	15	62	9	4*
5¾ hours	Glycogen only	0	0	0	...	...
	Chromatoids only ...	1	1	10	...	...
	Glycogen + chromatoids	0	0	0	...	...
	Neither	3	2	49	...	...
	Total	4	3	59	5	29*

* All contained four nuclei and none contained either glycogen or chromatoid bodies.

EXCYSTMENT OF *ENTAMOEBA HISTOLYTICA* IN CULTURES

On many occasions amoebae were actually observed to excyst: as already pointed out, the preparatory stages leading up to this appear to be multiplication of the nucleus and agglomeration of the four daughter nuclei, disappearance of the glycogen mass, and the formation and final disappearance of the chromatoid bodies. An individual which is about to excyst presents a characteristic appearance. The cytoplasm is more or less homogeneous and appears to be of a faintly-greenish tint, and is frequently very finely-alveolar; the nuclei in the living individual can be distinguished only with the

greatest difficulty. Careful examination shows that the amoeba is retracted in places from the cyst envelope and is evidently loose inside it; from time to time vigorous pseudopodial movements can be seen to take place (Pl. XXII, figs. 1 to 8). Finally, a rent apparently occurs in the cyst envelope, and a clear bead of ectoplasm is protruded; this progressively enlarges in a spasmodic manner, more and more of the amoeba protruding from the envelope, until finally the creature has escaped completely (Pl. XXII, figs. 9 to 15). It then proceeds to move about in an active, usually slug-like manner, frequently drawing behind it the empty cyst envelope or faecal débris. In the actively moving freshly-excysted *Entamoeba histolytica* the agglomerated nuclei are almost invariably to be found in the anterior part of the creature, and can easily be seen streaming into the pseudopodium immediately after this is protruded; the hindermost portion of the amoeba appears to take little part in active movement and seems to be dragged behind the advancing parasite as a sort of tail, and it is to this that the cyst envelope, or faecal débris, is often adherent (Pl. XXII, figs. 16 to 18). Although at the moment of emergence the cytoplasm is either practically homogeneous or, at most, very finely alveolar, with minute granules, it quickly becomes definitely alveolar, and as it ingests bacteria digestive vacuoles appear in large numbers.

For the study of living examples we employed the following method :—

The upper surface of a warmed microscope slide was coated with melted agar which was then allowed to solidify. The slide was then warmed to 37° C. and a drop of the material to be observed placed on it and covered with a slip, the under surface of which had been coated with agar in a similar manner; the preparation was carefully sealed with paraffin in order to prevent evaporation, and observed in the warm microscope chamber. Actual excystation can be better observed by mounting in this way material from a two-hour old culture of cysts on L.E.S. medium, than by using a suspension in Locke-serum of previously unincubated cysts.

The cyst envelope, after the escape of the amoeba, usually appears as a complete sphere with an extremely delicate wall; the rent through which the amoeba has emerged is very often invisible, but at times, by careful focussing and suitable illumination, it can be clearly seen (Pl. XXII, fig. 11). How long the cyst envelopes remain as such we cannot say, but we have found them in considerable numbers, frequently full of bacteria, in cultures twenty-four hours old.

DEVELOPMENT OF THE EXCYSTED AMOEBA

Our observations confirm, then, those of Penfold, Woodcock, and Drew, and of Chatton, that freshly-excysted *Entamoeba histolytica* are quadrinucleate, and suggest that Darling was mistaken when he described the division of the 4-nucleated encysted amoeba into four small amoebulae before excystment. As Chatton has pointed out, the nuclei of the quadrinucleate individuals are agglomerated together in a very characteristic manner. We cannot agree with Dobell (1919) that the nuclear agglomeration is, in this case, any indication of degeneration ; on the contrary, we believe it to be an absolutely constant and normal phenomenon. The subsequent development of the quadrinucleate amoebae is a matter of considerable interest ; at first we believed that they all quickly divided into uninucleate organisms, because these were found in large numbers in slightly older cultures. On examining, however, a fresh preparation of a culture about 16 hours old, we were much impressed with the relatively enormous size of a number of the amoebae, some of them, in fact, were of such gigantic dimensions that they occupied practically the entire microscope field ($\frac{1}{12}$ Obj., 4 Oc.). These individuals, although exhibiting considerable pseudopodial movement, did not, as a rule, show the fairly rapid translatory motion so characteristic of the ordinary amoebae. On examining them carefully it was seen that they were multinucleate, and on running iodine under the coverslip it was found that sometimes as many as 30 to 40, or occasionally even more, nuclei could be counted. These gigantic amoebae were discovered only in cultures of from about 10 to 30 hours old, and only in those originating from the inoculation of cysts (Pl. XXIV, figs. 1 to 6). We have never seen them in subcultures, or in cultures originating from the inoculation of vegetative amoebae from acute dysenteric stools. Consequently, it appears only reasonable to conclude that they are derived from the quadrinucleate excysted individuals, the nuclei of which continue to divide in the normal manner without a corresponding division of the cytoplasm. The proportion which these multinucleate individuals formed, varied considerably in different cultures ; generally they appeared to comprise but a comparatively insignificant percentage, but in some preparations they constituted as much as 20 to 24 per cent. of the total amoebae.

With a view to investigating the matter more closely, we performed a number of experiments which consisted in ascertaining, at stated intervals, the percentage of the various types of cyst and vegetative forms present in a culture, on L.E.S. medium, of a suspension of washed cysts; the preparations were stained with iodine. In these experiments, in order to avoid disturbing the cultures by frequent sampling, we inoculated a series of tubes so that each was examined on a single occasion only, and then discarded. As the experiment, which was repeated many times, on cultures made from cysts obtained from a number of different patients, always gave similar results, it will suffice if we consider but a single example, details of which are set forth in Table III.

TABLE III.

Showing the percentage of different types of cysts and of vegetative individuals, in cultures of various ages, made by sowing, on L.E.S. medium, cysts from a chronic dysenteric patient.

No. of hours incubated	Cysts					Vegetative amoebae				
	No. of nuclei			Nuclei indistinct, or cysts shrunken or granular	Cyst envelopes	No. of nuclei				
	1	2	4			1	2	3	4	Multi-nucleates
Original suspensions	57	18	15	10	...	...	...	...	...	...
2 hours ...	23	14	30	33	...	...	...	...	...	...
3½ hours ...	4	8	41	33	2	0	2	0	12	0
6½ hours ...	8	5	37	25	2	0	5	1	19	0
10½ hours	0	1	1	8	1	31	17	5	36	1
13 hours ...	1	0	3	10	2	48	18	5	12	3
16 hours ...	1	0	0	3	...	49	22	8	5	12
19 hours ...	1	0	2	3	...	63	15	6	2	8
24 hours ...	...	...	...	2	1	74	14	1	3	6
30 hours ...	...	...	1	1	...	86	5	0	2	5
33 hours ...	...	...	...	...	...	84	6	2	2	6
36 hours ...	...	...	...	...	...	90	5	2	0	3
48 hours ...	...	...	...	...	...	97	3	0	0	0

The developmental changes, which are disclosed in this table, are quite definite, and can be regarded as typical of what occurs in any L.E.S. culture of *Entamoeba histolytica* cysts. It will be observed that, in this particular case, the suspension of washed cysts, made from freshly-passed faeces, contained a marked preponderance of uninucleates : after 2 hours the cysts had so developed that the quadrinucleate individuals outnumbered the uninucleate. In $3\frac{1}{2}$ hours the uninucleates had decreased enormously in number and formed only a small fraction of the total cysts : at this period there was definite evidence of excystation, 14 per cent. of the individuals seen being vegetative forms. The $6\frac{1}{2}$-hour culture presented much the same appearance, except that the number of vegetative forms had increased to 25 per cent. of the total. These last two cultures afford conclusive proof that the freshly-excysted individuals are quadrinucleate ; no uninucleate forms were seen, and only comparatively few bi- and tri-nucleate individuals.

It will be observed that in the 2-hour, the $3\frac{1}{2}$-hour, and the $6\frac{1}{2}$-hour cultures there was a considerable increase of individuals falling into the ' Nuclei indistinct, or cysts shrunken or granular ' category ; undoubtedly some of these were dead or degenerate, but certainly many were forms in the stage immediately prior to excystment. As we have already pointed out, the contents of a cyst shortly before excystation become amoeboid and slightly withdrawn from the cyst envelope, and the nuclei are closely agglomerated. When such forms are stained by running iodine into the preparation, the shrinkage is often accentuated and the nuclear agglomeration, although it can be seen, becomes obscured and difficult to resolve into its four constituents. That this interpretation is correct is shown by the fact that in cultures over 6 hours old the number of individuals falling into the category in question had returned to about that existing in the original suspension. It must be admitted that in iodine-stained preparations of early cultures it is by no means easy to decide in all cases whether an individual is on the point of excysting, or whether it is dead and degenerate ; but it can be laid down as a general rule that in pre-excysting forms the cytoplasm is slightly alveolar and at most very finely granular, and the nuclear agglomeration can always be seen, although it is often somewhat indefinite and not clearly resolvable into its four constituents

(Plate XXI, figs. 19 and 20); whilst in the dead and degenerate forms the cytoplasm is much more coarsely granular and the nuclei are either distinct, and if more than one is present, separate, or else in advanced stages invisible; in such cases shrinkage of the contents from the envelope is often very pronounced (Plate XXI, figs. 21 to 24). In the 10½-hour old culture practically all the cysts have disappeared, except for a number obviously dead and degenerate; almost 90 per cent. of the individuals are vegetative forms, and whilst the quadrinucleate amoebae are still the most numerous, a relatively large number of uni-, and bi-nucleates are present, as well as a very few multinucleate (i.e., having more than four nuclei) forms. There is thus at this point evidence that some of the quadrinucleate amoebae are dividing into tri-, and bi-, and uninucleate individuals, and that in a few of them the nuclei are multiplying without division of the cytoplasm. In the next two cultures, 13 and 16 hours old, respectively, the decrease in number of the quadrinucleate amoebae, with a corresponding increase of uninucleates and multinucleates, is clearly seen. In still older cultures—19 to 48 hours—the proportion of uninucleate individuals steadily increases, whilst that of the multinucleates gradually dwindles to nothing.

Yoshida (1920) has described in freshly-excysted *Entamoeba tetragena*, and also in *Entamoeba coli*, fusion of the nuclei: this is stated to be of two types, the first a simple autogamy involving the fusion of two nuclei, and the second a polynuclear autogamy involving the fusion of several nuclei with the formation of a syncaryon. We have never been able to observe anything of this nature, either in fresh or in stained preparations. We believe the usual fate of the excysted quadrinucleate *Entamoeba histolytica* is its subdivision into four uninucleate amoebae, either by first dividing into two binucleate individuals, each of which again subdivides into two uninucleates, or probably most commonly by throwing off uninucleates one at a time. Plate XXIII, figs. 2 to 5 are camera lucida drawings of individuals from cultures 11 to 17 hours old, that is at a time when the quadrinucleate excysted amoebae were becoming converted into uninucleate individuals, and show the separation of one or two nuclei from the original agglomeration, a process which we believe to precede division of the quadrinucleate parasites.

UNDER WHAT CONDITIONS DOES *ENTAMOEBA HISTOLYTICA* EXCYST?

Having ascertained that *Entamoeba histolytica* rapidly excysts when sown on L.E.S. medium, and incubated at 37° C., and that under such circumstances a growth of vegetative forms is readily obtained, we decided to investigate whether excystation would take place under other conditions. It was found that when nutrient agar, or nasgar, was substituted for the egg medium, and the ordinary mixture of Locke's fluid (eight parts) and serum (one part) added, results exactly similar to those obtained on L.E.S. medium were observed; the cysts developed rapidly, excystment occurred, and the quadrinucleate amoebae divided into uninucleate, or formed multinucleate, individuals just as has been described in the experiments where L.E.S. medium was used.

In other experiments about 13 drops of suspension of washed *Entamoeba histolytica* cysts were added to tubes containing respectively 5 c.c. of Locke-serum, 5 c.c. of broth, or 5 c.c. of physiological saline. In all cases motile vegetative amoebae and cyst envelopes were seen after a few hours' incubation at 37° C.; furthermore, in each of the above fluids actual excystment was observed. The cysts themselves gradually disappeared, and after 24 to 36 hours nothing but a few obviously degenerate cysts was to be seen. Although the excysted amoebae could be observed to be moving actively for a time, they failed to multiply as on the L.E.S., Agar L.S., or Nasgar L.S. media, and soon died.

When 13 drops of the suspension of washed cysts were added to 5 c.c. of water and incubated at 37° C., definite changes occurred, which, so far as they went, were quite comparable with those recorded above. The majority of the uninucleate cysts became quadrinucleate just as on any of the nutrient media. No active vegetative amoebae were found, but occasional cyst envelopes were seen and sometimes the initial stages of excystment. Our observations left us in no doubt that, in many cysts, the normal development continued so far as rupture of the cyst-envelope: when this happened the amoebae were quickly killed by the water; as a rule the creature died before escaping from the envelope, sometimes it died when partly excysted, and sometimes it actually escaped from the envelope before perishing.

It seems quite clear that the suspension of the cysts in water at 37° C. is not deleterious to the cysts, but only to the excysting amoebae ; subcultures made on L.E.S. medium, from the water suspension after 4 or 6 hours' incubation, resulted in a good growth of vegetative amoebae.

So far as we have been able to ascertain the conditions essential for the development and excystation of *Entamoeba histolytica* are moisture and a suitable temperature, preferably about 37° C.

SUMMARY

1. *Entamoeba histolytica* cysts develop and excyst under suitable conditions *in vitro*.

2. So far as we have been able to ascertain, moisture and a suitable temperature (preferably about 37° C.) are essential for the occurrence of excystation ; the passage of the cysts through such solutions as *liquor pepticus* or *liquor pancreaticus* is unnecessary for excystation.

3. The changes attending development and excystation can readily be followed in cultures of *Entamoeba histolytica* cysts on L.E.S. and certain other media at 37° C.

4. Great variation in the stage of development of *Entamoeba histolytica* cysts may be seen in the freshly-passed faeces of different individuals, and of the same individual at different times. These variations concern chiefly the number of nuclei, the glycogen content, and the percentage containing chromatoid bodies.

5. The youngest cysts are uninucleate and loaded with glycogen ; as development takes place chromatoid bodies make their appearance, the nuclei divide, and the glycogen decreases in amount. Later, the cyst becomes quadrinucleate ; chromatoid bodies are well-developed, but the glycogen is much less evident, or entirely absent. When the cyst is completely mature, and ready to excyst, the nuclei are agglomerated and the cytoplasm is homogeneous and without glycogen ; the chromatoid bodies are greatly reduced or absent.

6. We have failed to observe any evidence of autogamy in the development of the cysts, but we have frequently seen chromatin particles apparently extruded from the nuclei immediately prior to, or during, division.

7. The freshly-excysted *Entamoeba histolytica* contains four closely agglomerated nuclei, and the cytoplasm is very finely-alveolar. It moves in a characteristic slug-like manner, with the nuclei almost invariably anterior, and drags behind a more or less motionless tail; as bacteria are ingested the cytoplasm becomes more alveolar and digestive vacuoles quickly appear.

8. In cultures the majority of the quadrinucleate excysted amoebae divide either directly, or indirectly, into four uninucleate individuals, but in a certain proportion the cytoplasm fails to divide and multinucleate individuals are formed. Here again we were unable to observe any indication of autogamy.

9. A method of obtaining concentrated washed suspensions of *Entamoeba histolytica* cysts is described. Inoculation on L.E.S., or other suitable, medium with such suspensions is the simplest way of obtaining excellent cultures of vegetative forms.

REFERENCES

Boeck, W. C. (1924). Studies on *Tricercomonas intestinalis* (Wenyon and O'Connor, 1917). *Amer. Jl. Trop. Med.*, Vol. IV, No. 6, p. 519.

Boeck, W. C., and Drbohlav, J. (1925). The Cultivation of *Endamoeba histolytica*. *Amer. Jl. Hyg.*, Vol. V, No. 4, p. 371.

Chatton, E. (1917). L'éclosion des kystes et les premiers stades de l'évolution de l'amibe dysentérique humaine chez le chat. *Bull. Soc. Path. Exot.*, Vol. X, No. 9, p. 834.

Cutler, D. W. (1919). Observations on *Entamoeba histolytica*. *Parasitology*, Vol. XI, No. 2, p. 127.

Darling, S. T. (1913). Observations on the Cyst of *Entamoeba tetragena*. *Archiv. Int. Med.*, Vol. XI, No. 1, p. 1.

Dobell, C. (1919). The Amoebae living in Man: a zoological monograph. London.

Dobell, C., and O'Connor, F. W. (1921). The Intestinal Protozoa of Man. London.

Hoare, C. A. (1925). Exhibit at Laboratory Meeting, 19th November, 1925. *Trans. Roy. Soc. Trop. Med. & Hyg.*, Vol. XIX, Nos. 5 and 8, p. 277.

Penfold, W. J., Woodcock, H. M., and Drew, A. H. (1916). The Excystation of *Entamoeba histolytica* (*tetragena*) as an indication of the vitality of the cysts. *B.M.J.*, Vol. I, p. 714.

Sellards, A. W., and Theiler, M. (1924). Investigations concerning Amoebic Dysentery. *Amer. Jl. Trop. Med.*, Vol. IV, No. 3, p. 309.

St. John, J. H. (1926). Practical value of examination for *Endamoeba histolytica* by culture. *Jl. Amer. Med. Assoc.*, Vol. LXXXVI, No. 17, p. 1272.

Ujihara, K. (1914). Studien über die Amöbendysenterie. *Zeitsch. f. Hyg.*, Vol. LXXVII, p. 329.

Wenyon, M. (1907). Observations on the Protozoa in the Intestine of Mice. *Archiv. f. Protist.*, Supplement I, p. 169.

Yoshida, K. (1920). Reproduction *in vitro* of *Entamoeba tetragena* and *Entamoeba coli* from the cysts. *Jl. Exp. Med.*, Vol. XXXII, No. 3, p. 357.

EXPLANATION OF PLATE XXI

All figures are camera lucida drawings of *Entamoeba histolytica* cysts stained with iodine; magnification about 1,730. The figures represent the various stages in the development of the cysts as seen either in freshly-passed faeces or in cultures.

Fig. 1. Uninucleate cyst with a large glycogen mass.

Fig. 2. Uninucleate cyst with a glycogen mass and a chromatoid body.

Figs. 3 and 4. Uninucleate cysts showing fragmentation of the karyosome and extrusion of chromatin particles.

Figs. 5 to 8. Various stages in the division of the nucleus in uninucleate cysts.

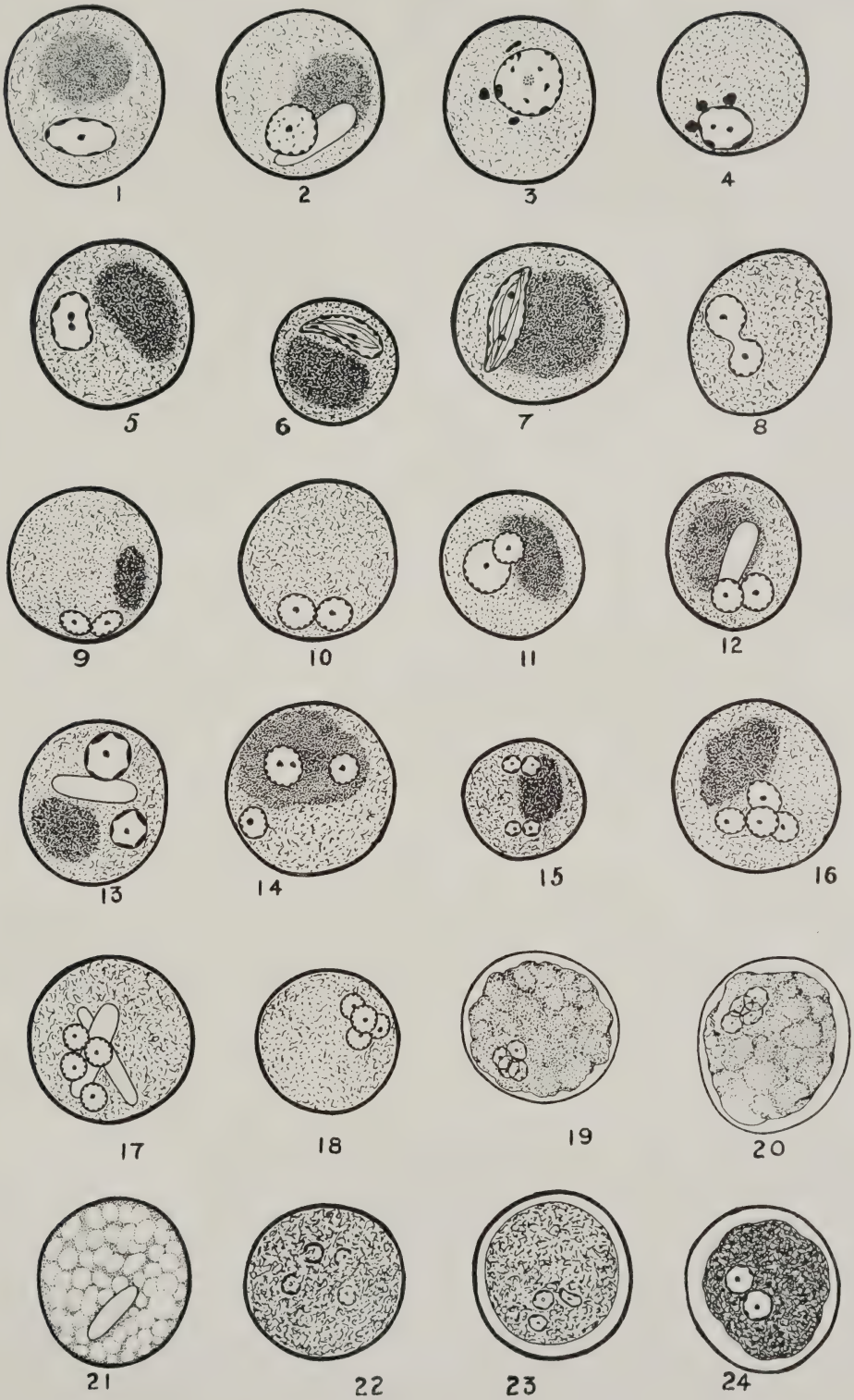
Figs. 9 to 13. Various types of binucleate cysts.

Fig. 14. Trinucleate cyst.

Figs. 15 to 18. Various types of quadrinucleate cysts.

Figs. 19 and 20. Pre-excysting forms: these individuals were actually seen to be moving within the cyst-envelope before iodine was run into the preparation.

Figs. 21 to 24. Various dead and degenerate cysts.



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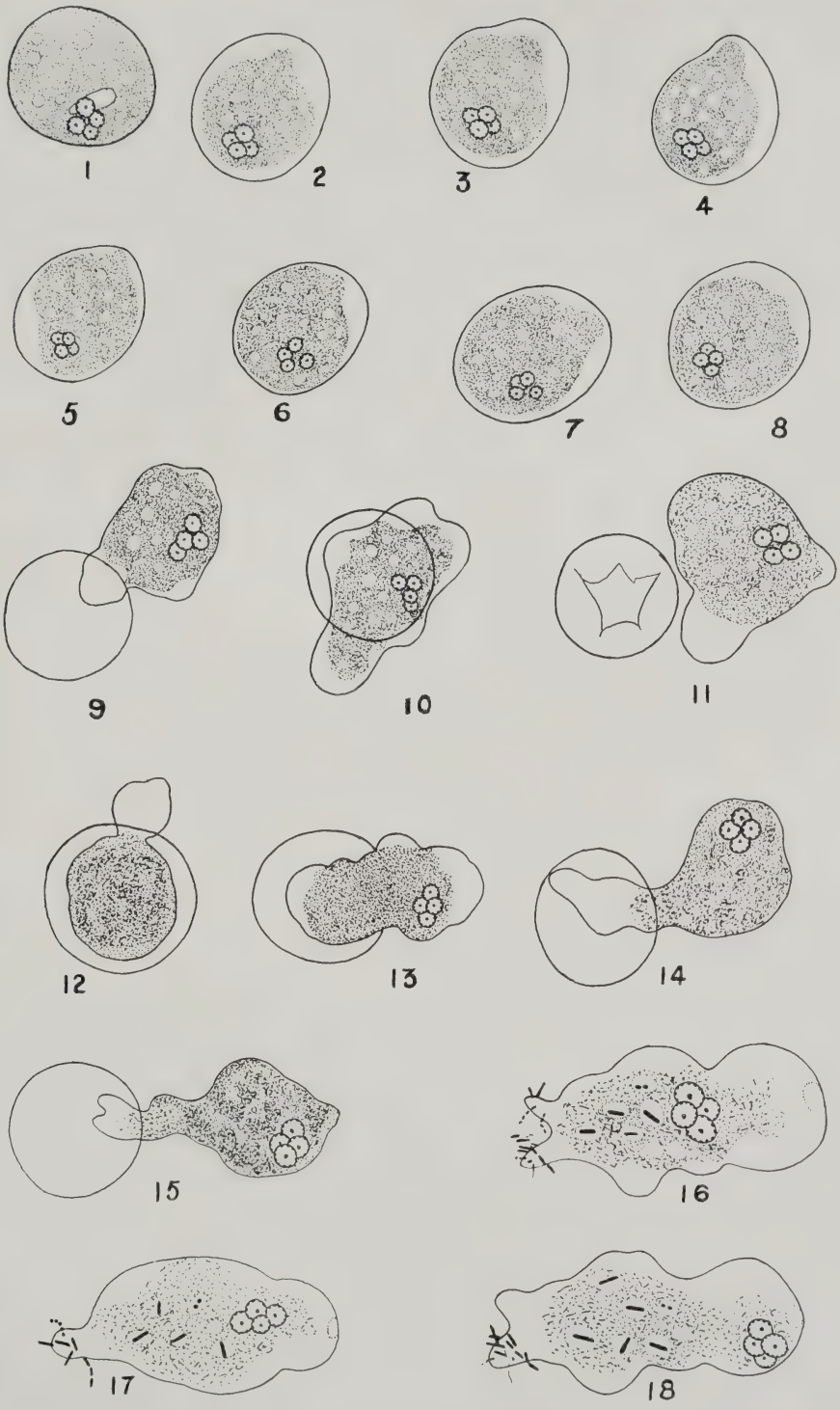
EXPLANATION OF PLATE XXII

All figures are camera lucida drawings of living specimens of *Entamoeba histolytica :* magnification about 1,500.

Figs. 1 to 11. Showing the successive changes occurring in a single cyst kept at 37° C. and observed over a period of about an hour ; Fig. 1, a mature 4-nucleated cyst with the remnant of the chromatoid body and agglomeration of the nuclei ; Figs. 2 to 8, showing the total disappearance of the chromatoid body, the retraction of the cytoplasm from the cyst envelope, and the amoeboid movements of the parasite within the envelope ; Figs. 9 to 11, the escape of the amoeba from the cyst envelope.

Figs. 12 to 15. Various stages in excystation observed in the case of another cyst.

Figs. 16 to 18. A recently excysted amoeba showing the agglomerated nuclei following closely the advancing pseudopodium, and the more or less motionless ' tail ' to which are attached bacteria and débris.



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EXPLANATION OF PLATE XXIII

All figures are camera lucida drawings of iodine-stained preparations of *Entamoeba histolytica* as seen in 10 to 20 hour-old cultures of cysts. Magnification about 1,500.

The figures show what we believe to represent the usual fate of the quadrinucleate excysted amoebae, viz., their division into tri-, bi-, and finally, uni-nucleate individuals.

Fig. 1. Typical quadrinucleate amoeba showing nuclear agglomeration.

Figs. 2 to 4. Amoeba showing separation of one nucleus from the agglomeration, presumably preparatory to the separation from the parent of a uninucleate individual.

Fig. 5. Showing simultaneous separation of two of the nuclei from the agglomeration.

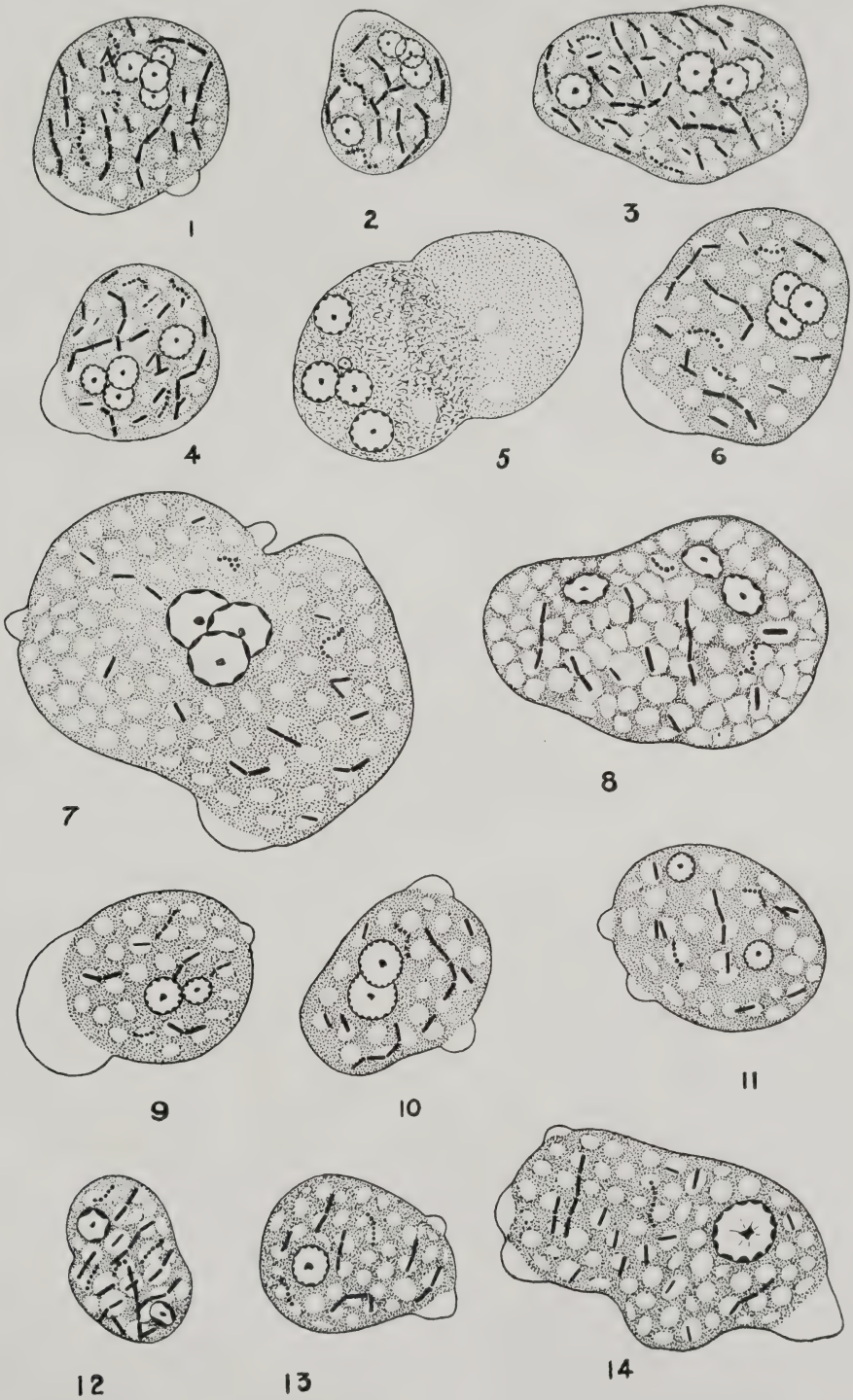
Figs. 6 and 7. Trinucleate amoebae with agglomerated nuclei.

Fig. 8. Separation of one of the nuclei in a trinucleate amoeba.

Figs. 9 and 10. Binucleate amoebae with agglomerated nuclei.

Figs. 11 and 12. Separation of the nuclei of binucleate amoebae preparatory to division.

Figs. 13 and 14. Uninucleate amoebae.



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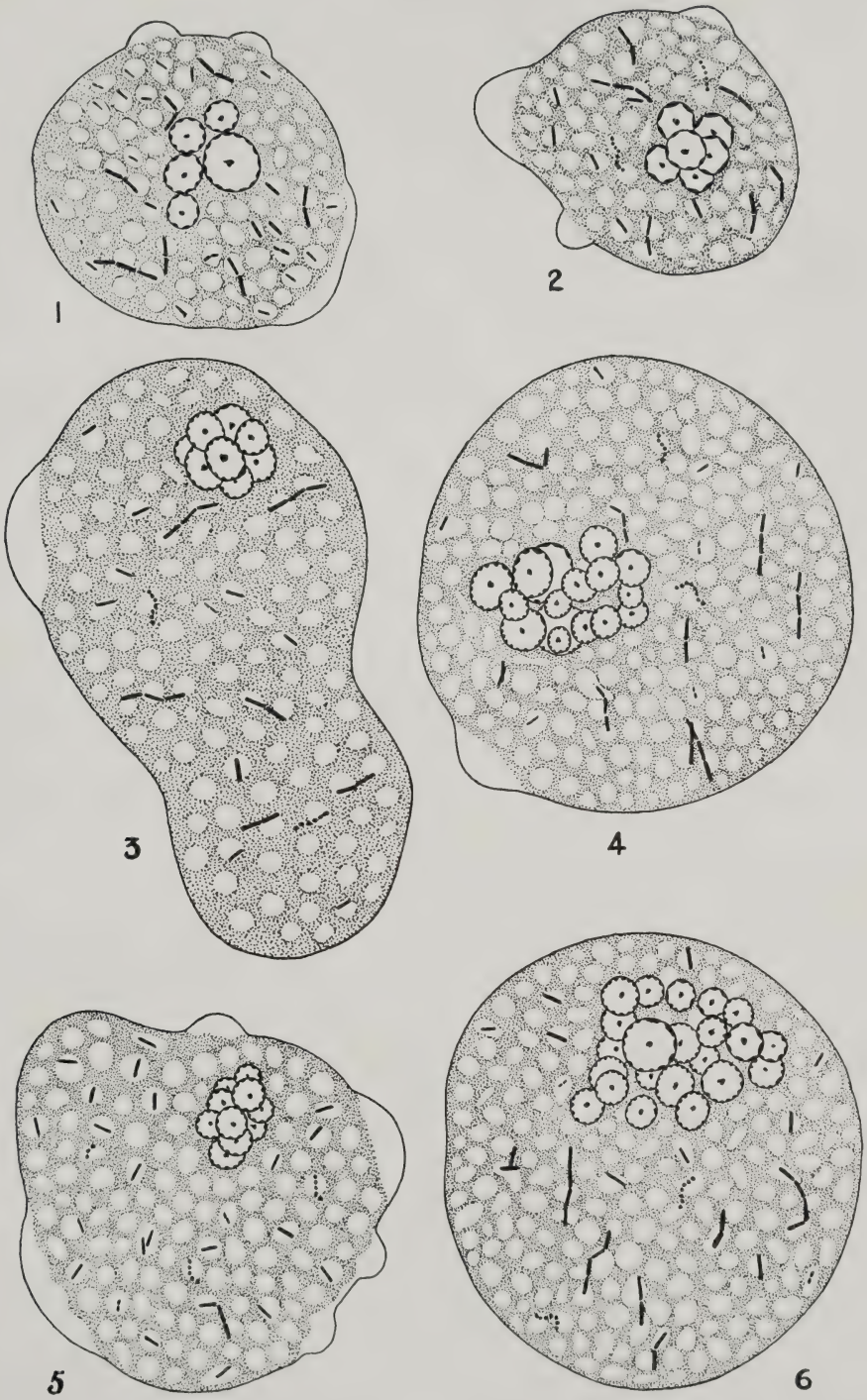
EXPLANATION OF PLATE XXIV

All figures are camera lucida drawings of iodine-stained preparations of *Entamoeba histolytica* as seen in 10 to 20 hour-old cultures of cysts. Magnification about 1,500.

Figs. 1 to 6. Typical examples of multinucleate amoebae.



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